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In Situ Survival of *Vibrio cholerae* and *Escherichia coli* in a Tropical Rain Forest Watershed

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For 12 months, *Vibrio cholerae* and fecal coliform densities were monitored along with nine other water quality parameters at 12 sites in a rain forest watershed in Puerto Rico. Densities of *V. cholerae* and fecal coliforms were not significantly correlated, even though the highest densities of both bacteria were found at a sewage outfall. High densities of *V. cholerae* were also found at pristine sites at the highest point in the watershed. The density of *Escherichia coli* and *V. cholerae* in membrane diffusion chambers did not change significantly during the course of two such studies. Physiological activity, as measured by electron transport system activity and relative nucleic acid composition, indicated that both *E. coli* and *V. cholerae* remained active. This study suggests that *V. cholerae* is indigenous to tropical fresh waters and that assays other than those that detect fecal coliforms or *E. coli* must be used for assessing public health risk in tropical waters.

Recent in situ studies by our laboratory in Puerto Rico, using direct enumeration and activity measurements, have shown that *Vibrio cholerae* can survive and remain moderately active on a tropical coral reef (14). In Puerto Rico and other parts of the Caribbean, *V. cholerae* has even been isolated from near-shore coastal waters when the salinity was 35‰ and the temperature was 25°C (3, 7, 21). Fresh water in India was also shown recently to harbor very high densities of *V. cholerae* (13). These findings have suggested that tropical rain forest environments should provide ideal conditions for *V. cholerae* survival, yet few if any studies have examined the survival of this pathogen in this environment. Indeed, considering the importance of this pathogen in underdeveloped tropical nations, an understanding of the survival of *V. cholerae* in this environment is vital to tropical public health. In the tropics, coliforms and fecal coliforms are universally used as indicators of the presence of pathogens (8). The target enteric organism in both of these assays is *Escherichia coli*. Thus, it is essential that we examine the survival of *E. coli* along with that of *V. cholerae*.

(This study was part of the M.S. thesis of N. Pérez-Rosas at the University of Puerto Rico, Río Piedras, Puerto Rico, 1983.)

The study site was the Mameyes River watershed located on the northeastern coast of the island of Puerto Rico at 18° 15′ N, 65° 45′ W (Fig. 1). (For detailed descriptions of the study area, see Carrillo et al. [4], López-Torres et al. [10], and Valdés-Collazo et al. [22].)

Measurements were taken in situ for conductivity, salinity, pH, dissolved oxygen, light intensity, and temperature. Turbidity, alkalinity, hardness, light intensity, and ammonia concentration were measured in the field. Chlorophyll *a*, nitrate plus nitrite, sulfate, total phosphorus, and P_i were measured by using procedures recommended in *Standard Methods for the Examination of Water and Wastewater* (1). All measurements were as described before (4, 11, 14, 22).

For *V. cholerae* enumeration, water samples were filtered through 0.45-μm-pore-size membrane filters (Millipore Corp., Bedford, Mass.). Filters were then placed on thiosulfate-citrate-bile salts-sucrose agar (Difco Laboratories, Detroit, Mich.) and incubated at 37°C for 24 h. All round, yellow colonies were considered *V. cholerae*-like (1). Identities of random isolates were confirmed by using biochemical tests (API 20E strips; Analytab Products, Plainview, N.Y.). *V. cholerae* identities were confirmed by using polyvalent antiserum against *V. cholerae* (Difco). Fecal coliform densities were determined by filtering samples with type HC, 0.7-μm-pore-size membrane filters (Millipore). Filters were placed on mFC media (Difco) in tight-fitting petri dishes (Millipore) and incubated at 44.5 ± 0.1°C in a block-type FC incubator (Millipore) for 24 h. All blue colonies were considered fecal coliform positive and enumerated (1).

Direct cell counts for *V. cholerae* and *E. coli* in diffusion chambers were done by using a Coulter Counter (Coulter Electronics, Inc., Hialeah, Fla.) and by acridine orange direct counting (AODC). All counts were as described before (4, 10, 11). The total number of bacteria and the number involved in respiration were determined by the 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenytetrazolium chloride (INT) reduction technique of Zimmermann et al. (25).

For survival studies, Plexiglas diffusion chambers, each with a 100-ml capacity, were used with 0.45-μm-pore-size, nylon-reinforced Versapor membrane filters (Gelman Instrument Co., Ann Arbor, Mich.) as the diffusion surface. Pure cultures of *V. cholerae* and *E. coli* were placed into each sterile diffusion chamber just before it was placed at the study site. All preparations and sample collections were as described before (4, 10, 11). Two separate chamber studies were conducted several months apart; study A compared sites 4 and 5, while study B compared sites 1 and 4. Activity was measured during study A only, because of resource limitations.

The data were analyzed by using prepared programs for Apple II, Macintosh, and IBM 4321 computers. Factorial

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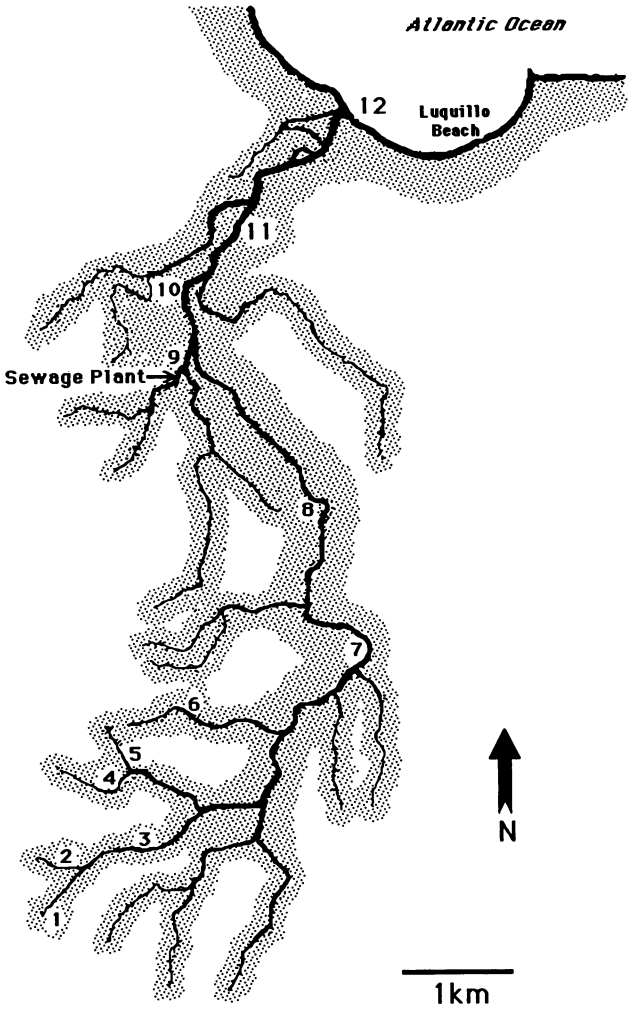

FIG. 1. Locations of study sites in the Mameyes River watershed, Puerto Rico.

analyses of variance were used to test for differences among sites and collection times. Data were subjected to the appropriate transformation before statistical analysis by the method of Zar (24). Any probability less than or equal to 0.05 was considered significant.

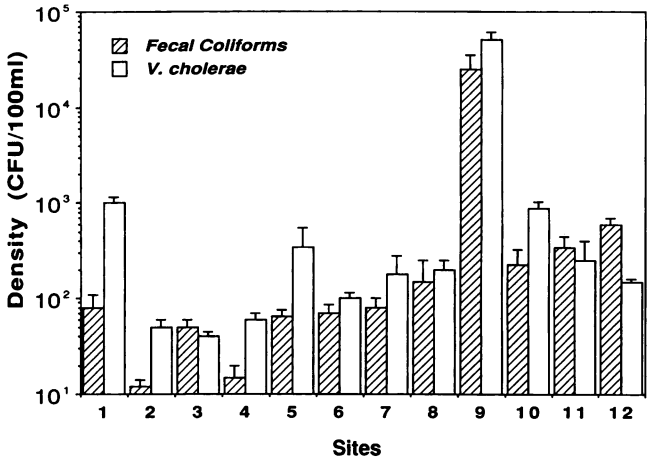

FIG. 2. Densities of *V. cholerae* and fecal coliforms by site in the Mameyes River watershed.

During the sampling period, the physicochemical parameters measured showed little variability within the same sites (Table 1). The Mameyes River watershed is relatively oligotrophic, despite the fact that the source of the watershed is a cloud-type tropical rain forest. (For a complete description of all sites and a thorough discussion of the trophic status of this watershed, see Carrillo et al. [4], López-Torres et al. [10], and Valdés-Collazo et al. [22].) Torrential rainfalls (>10 cm/h), which can cause the river level to change more than 2 m in less than 1 h, are the principal cause of the low densities of resident flora and fauna and the lack of seasonality in all parameters (C. F. Aranda, M.S. thesis, University of Puerto Rico, Río Piedras, 1982). Temperature is quite constant year-round, and rainfall does not exhibit any consistent pattern in the area. The low variability in water quality parameters would not allow significant correlations with bacterial densities.

Densities of both *V. cholerae* and fecal coliforms differed significantly by site (Fig. 2). Densities of *V. cholerae* and fecal coliforms were not significantly correlated when compared on the basis of site, time, or both site and time. The highest densities of fecal coliforms and *V. cholerae*, as expected, were recorded at site 9, the sewage outfall. However, densities of fecal coliforms reported at other sites in this study were lower than those reported by Evison and James (5) for river samples taken in two countries in tropical

TABLE 1. Water quality of Río Mameyes (Mameyes River), Puerto Rico[a]

Site	ATEMP (°C)	WTEMP (°C)	DO (mg/liter)	pH	Sal (‰)	NO_{2+3} (mg/liter)	PO_4 (μg/liter)	TP (μg/liter)	ChlA (mg/liter)
1	23 ± 1.0	21 ± 0.5	7.9 ± 0.2	6.2 ± 0.1	0	0.4 ± 0.1	2.4 ± 0.9	3.5 ± 1.2	148 ± 71
2	23 ± 1.0	20 ± 0.5	6.9 ± 1.1	6.3 ± 0.1	0	0.4 ± 0.4	0.9 ± 0.3	2.8 ± 1.8	22 ± 21
3	23 ± 0.9	20 ± 0.5	7.8 ± 0.5	6.5 ± 0.2	0	0.4 ± 0.4	1.47 ± 0.5	4.8 ± 2.5	9 ± 7
4	23 ± 0.9	22 ± 0.9	8.1 ± 0.2	6.8 ± 0.1	0	0.7 ± 0.2	1.0 ± 0.7	5.7 ± 2.8	47 ± 19
5	24 ± 0.9	22 ± 0.4	8.2 ± 0.2	7.2 ± 0.1	0	0.5 ± 0.1	1.4 ± 0.9	3.0 ± 1.2	77 ± 29
6	25 ± 1.0	21 ± 0.3	7.5 ± 0.7	7.0 ± 0.2	0	0.2 ± 0.1	2.3 ± 0.7	6.9 ± 6.0	18 ± 17
7	26 ± 1.0	23 ± 0.5	7.3 ± 1.2	7.0 ± 0.1	0	0.4 ± 0.3	6.7 ± 3.5	5.3 ± 3.8	30 ± 29
8	27 ± 1.0	24 ± 0.8	7.2 ± 0.6	7.1 ± 0.1	0	0.5 ± 0.4	3.0 ± 0.8	2.9 ± 0.6	63 ± 61
9	28 ± 1.4	25 ± 0.9	6.9 ± 0.5	7.0 ± 0.1	0	1.4 ± 0.7	6.8 ± 2.3	9.0 ± 2.6	106 ± 63
10	29 ± 2.0	25 ± 1.0	7.4 ± 1.0	7.1 ± 0.2	0	0.1 ± 0.0	4.8 ± 0.9	10 ± 1.4	44 ± 42
11	29 ± 2.0	26 ± 1.0	7.2 ± 1.0	7.2 ± 0.2	0	0.3 ± 0.2	6.8 ± 0.9	2.3 ± 1.0	63 ± 22
12	30 ± 2.0	25 ± 0.3	6.2 ± 1.0	7.4 ± 0.2	5.0 ± 3.0	0.2 ± 0.1	3.6 ± 1.9	8.6 ± 3.9	54 ± 53

[a] All values are the mean ± 1 standard error (n = 6). Abbreviations: ATEMP, air temperature; WTEMP, water temperature; DO, dissolved oxygen concentration; Sal, salinity; NO_{2+3}, nitrites plus nitrates; PO_4, P_i; TP, total phosphorus concentration; ChlA, chlorophyll *a* concentration.

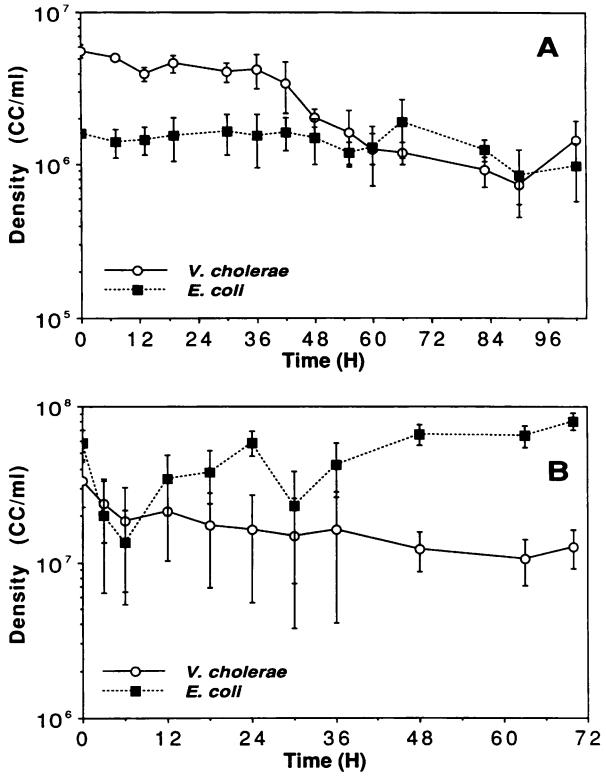

FIG. 3. Changes in total density as measured with a Coulter Counter for *V. cholerae* and *E. coli* for study A (sites 4 and 5) (A) and study B (sites 1 and 4) (B) (mean ± 1 standard error; $n = 8$).

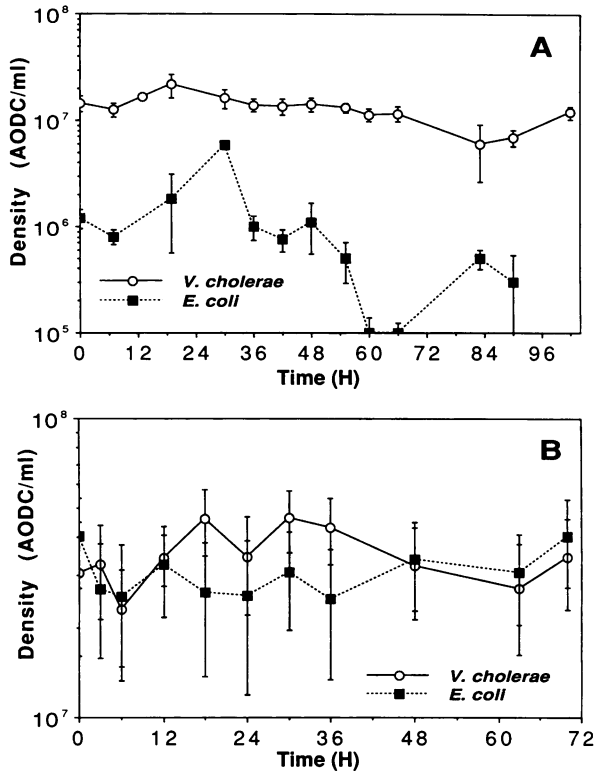

FIG. 4. Changes in total density as measured by AODC for *V. cholerae* and *E. coli* for study A (sites 4 and 5) (A) and study B (sites 1 and 4) (B) (mean ± standard error; $n = 8$).

Africa and much higher than those reported for river samples taken in England.

Densities of *V. cholerae* were also high at site 1, a pristine area high in the rain forest (Fig. 2). As noted in other studies, however, this locale has higher densities of fecal coliforms, total anaerobes, and *Candida albicans* than sites immediately below it in the watershed (4, 18, 22). Densities of *V. cholerae* and fecal coliforms were not significantly correlated when compared on the basis of site, time, or both site and time. Recent studies have even demonstrated that supposedly anthropogenic microorganisms such as *E. coli* may be indigenous in this environment (2, 16). High densities of *V. cholerae* have also been reported in tropical fresh waters in India (13). Indeed, several investigators have reported the isolation of *V. cholerae* in cold, uncontaminated, temperate fresh waters (12, 15).

Direct-count density estimates for both *V. cholerae* and *E. coli* changed little during the course of two studies in the upper part of the watershed (Fig. 3 and 4). In fact, the calculated time to 90% reduction (T_{90}) for both bacteria for both studies was infinite, except for AODC densities of *E. coli* in study A (T_{90} = 108 h). This one anomaly is readily explained by the variability in density observed over time and the density increases observed from 6 to 30 h and from 66 to 84 h. West and Lee (23) in England also observed stable survival of *V. cholerae* in diffusion chambers during the summer months, when river water reached its highest annual temperature.

The survivability of *V. cholerae* and *E. coli* in this environment is also borne out by the activity measured during study A (Fig. 5). Though the percentage of respiring *V. cholerae* cells declined during the first 18 h by more than 80%, it increased during the next 48 h and then stabilized at 78%; this increase is indicative of a very active population ($F = 2.18$, df = 17 and 104, $P < 0.05$; Fig. 5B). As reported by Zimmermann et al. (25), natural populations of bacteria in cold waters rarely exceed 10% activity in INT reduction. This finding is confirmed by the differential fluorescence of acridine orange, which indicated that more than 90% of the cells were active at all times of the study. It is assumed that INT reduction indicates cells actively respiring and that acridine orange fluorescence indicates proportions of RNA and DNA, i.e., protein synthesis. Thus, *V. cholerae* cells in the chambers initially declined in respiration rate and subsequently acclimated their respiring ability in response to the new environment to which they were exposed; however, the stress of the new environment was not sufficient to reduce protein synthesis during the acclimation period. This suggests that fermentative metabolism may have been able to compensate for the loss in oxidative metabolism. *Klebsiella pneumoniae* shows a similar pattern of activity response in this environment (10).

The activity of *E. coli* as measured by both acridine orange fluorescence and INT reduction was significantly lower than that of *V. cholerae*, indicating a more stressful environment for *E. coli* (Fig. 5). The variability for both activity measurements was also much greater for *E. coli*. However, even at these lower measurements of activity for *E. coli*, the activities observed were greater than those observed for natural populations of bacteria in both warm (20) and cold fresh waters (25). Indeed, three other studies at these same sites have also demonstrated that *E. coli* can survive and remain

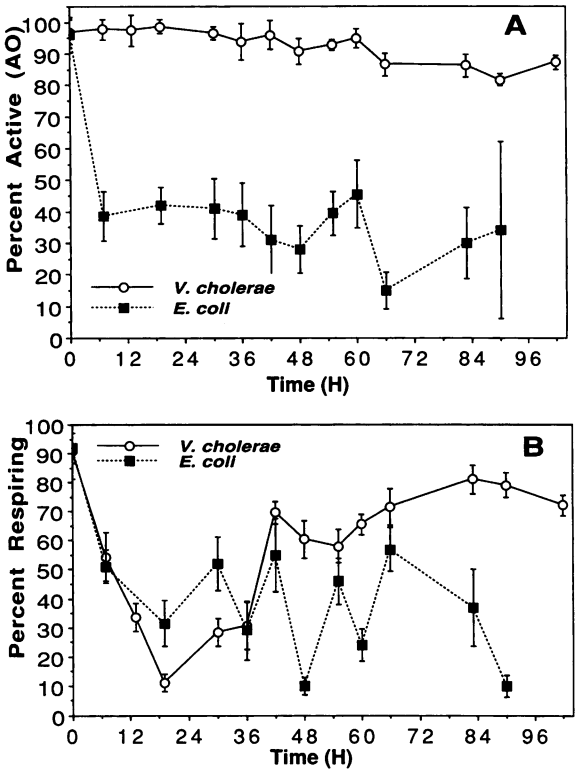

FIG. 5. Changes in percent activity as measured by AODC (A) and INT reduction (B) for *V. cholerae* and *E. coli* for studies A and B (sites 4 and 5) (mean ± 1 standard error; $n = 8$).

active in this environment for extended periods of time (4, 10, 22).

The diffusion chamber study of West and Lee (23) in an English river, the mathematical model of Seidler and Evans (19), and the surveys of Roberts et al. (17) in Louisiana all indicate that high temperatures and low salinities are conducive to *V. cholerae* survival. The present study confirms that tropical fresh waters are nearly ideal for *V. cholerae* and may represent one of its natural habitats. The lack of correlation between fecal coliforms and *V. cholerae* found in this study and found by several other investigators suggests that assays for fecal coliforms are not reliable indicators of pathogens such as *V. cholerae* (9, 17). The ability of both *V. cholerae* and *E. coli* to survive and remain active in tropical environments further suggests the unreliability of such assays for indicating recent human fecal contamination and thus public health risk. The increased survival and possible indigenous nature of pathogens such as *V. cholerae* further emphasize the need for direct enumeration standards and health risk assessments for underdeveloped tropical countries, where these diseases exact a horrible toll.

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